Biochemical and Physiological Effects of Chlordimeform

by F. Matsumura* and R. W. Beeman*

Chlordimeform is a relatively new acaricide/insecticide, whose mode of action we have investigated. It appears to interfere with amine-mediated control of nervous and endocrine systems in a variety of ways. Specifically, chlordimeform causes a build-up of the amines 5-hydroxytryptamine and to a lesser extent norepinephrine in the rat brain *in vivo*, antagonizes the *in vivo* action of reserpine in the rat (reserpine depletes amine stores in the CNS), inhibits monoamine oxidase from rat liver *in vitro*, and causes hypotension in rabbits. In the American cockroach it directly stimulates the heart *in situ*, acts synergistically with tryptamine *in vivo*, inhibits amine-*N*-acetyltransferase from cockroach head *in vitro*, causes accumulation of indolamines in cockroaches *in vivo*, and blocks the stimulation of adenylate cyclase by octopamine in the cockroach CNS *in situ*. It also inhibits tryptamine metabolism in whole mites *in vitro*.

Chlordimeform is a very unusual pesticide from several standpoints. First, its effectiveness against insect and acarine pests in the field does not always come from its direct killing action. For instance, chlordimeform causes marked excitation in adult rice stem borers which results in abnormal egg-laying behavior. In the young larvae, chlordimeform causes different types of behavioral changes which may be collectively described as general sedation. Here the larvae are inactive and therefore do not find proper sites for boring. Affected larvae often do not drill holes into the stem, and, even if they enter the stem, seldom eat. Chlordimeform also has some repellent action, and hence some larvae have been observed to move away from the normal food source. Second, chlordimeform exhibits a remarkable selectivity to a few groups of arthropods, namely to lepidopterous insects and acarine species. Even within the lepidopterous insects its effectiveness is often confined to mature eggs, young larvae, and in limited instances to adults. Such a selectivity pattern has never been observed in other classes of insecticides-acaricides. Third, chlordimeform causes very different symptoms in

different animal species and stages. In addition to the above example of the rice stem borer, where adults show an entirely different poisoning symptom from the larvae, we know that the symptoms elicited by one mammalian species can differ from those shown by another species. Cows which are treated with chlordimeform for cattle tick control are known to show marked sedation, while chlordimeform-treated dogs exhibit symptoms of excitation.

Fourth, chlordimeform is effective against many types of mites and insects resistant to conventional pesticides, such as organophosphates and chlorinated hydrocarbon insecticides *(1)*. This should mean that the defense mechanisms acquired by these resistant populations are useless against chlordimeform, indicating that it is likely to have an entirely different action mechanism from those conventional pesticides.

From the above description it must be apparent that our interest in chlordimeform has grown out of our suspicion that its action mechanism was new. This possibility of a novel poisoning mechanism suggests new areas of research involving studies on hitherto unsuspected weaknesses of these pest species as well as its biochemical and physiological effects on mammalian species.

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Results

Studies in Higher Animals

Acute Toxicity and Poisoning Symptoms: The acute toxicity of chlordimeform to mammals is relatively low. By our estimation the acute, intraperitoneal LD_{50} values for rats is 200 mg/kg. Acute oral and intraperitoneal LD_{50} values for mice and rabbits are also estimated to be of the same order of magnitude (CIBA information sheet).

At a high dose (200 mg/kg, IP) chlordimeform causes marked hyperexcitation in a short time period (5 to 10 min) in rats and mice. They exhibit tremors and become extremely hypersensitive to external stimuli. The poisoned animals show signs of locomotive difficulties, partly due to frequent hyperextension of hind legs. It is important to note here that the death, if it is going to occur, almost always takes place in this period of hyperexcitation at a very early stage (1–3 hr). In other words, the animals which can withstand this eventually recover. In rats at all doses tested (50–200 mg/kg), gradual dilation of pupils took place over a 1-hr period. Throughout the entire duration of this early excitation period the animals have not been observed to show cholinomimetic symptoms as slowing of the heart beat, salivation, urination or fasciculation.

Following these initial periods of hyperexcitation the animals gradually fall into a state of sedation. The transition can be clearly recognized, since they no longer make attempts to run around. At the midst of the sedation period, the poisoned rats, when placed in an open area, no longer show the escape reaction to a nearby corner, or a shelter. Instead, they stay motionless in a characteristic low posture unless they are externally stimulated. The state of sedation induced by chlordimeform differs from the one induced by general sedatives such as phenobarbital, in that in the former case the animal remains alert to external stimuli such as clapping of hands, showing quick jumping and running responses.

At lower doses (50–200 mg/kg) the duration of the initial excitation period becomes shorter, and in some instances the animal shows no sign of excitation. In such animals intermittent periods of "sedation" occur over a 1-hr period.

The recovery occurs gradually and in most cases, the animals behave seemingly normal within 24 hr.

Physiological and Biochemical Effects in Vivo: An interaperitoneal injection of 200 mg/kg of chlordimeform into rabbits caused a marked decrease in mean arterial pressure (carotid artery) of almost 50% within 30 min of injection (Fig. 1). To study the change in amine levels male rats were first treated with 200 mg/kg (IP) of chlordimeform, were killed after 1 hr, and their brains quickly removed. The serotonin and norepinephrine levels in the whole brain were then measured by the method of Maickel et al. (2). The result, shown in Table 1, indicates that the amine levels, particularly that of serotonin, were noticeably high in the brains of chlordimeform treated rats.
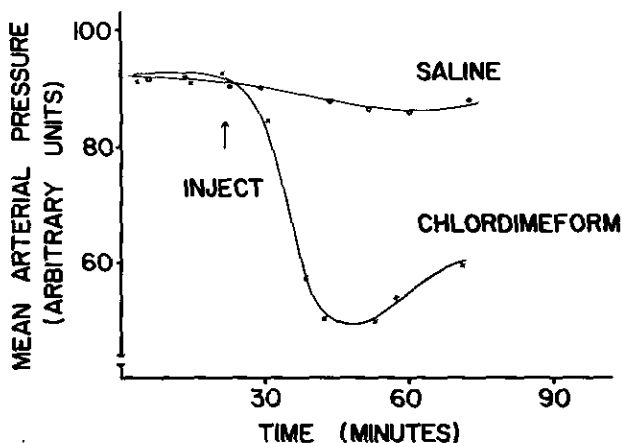

FIGURE 1. Effect of chlordimeform on the mean arterial pressure of the anesthetized rabbit. Male rabbits weighing 1.4–4.5 kg were anesthetized with 1 mg pentathol, IV, and anesthesia was maintained with nembutal. The carotid artery was cannulated, and the mean arterial pressure was monitored continuously. Saline or chlordimeform hydrochloride (200 mg/kg, 50 mg/ml) was injected IP at the time indicated by the arrow.

Table 1. Serotonin and norepinephrine levels in whole rat brain.[a]

	Chlordimeform	Control
Serotonin, $\mu g/g$ wet weight	0.75 ± 0.07	0.44 ± 0.06
Norepinephrine, $\mu g/g$ wet weight	0.22 ± 0.01	0.18 ± 0.03

[a]Data expressed as levels ± standard deviation. Averages of six animals each for serotonin and three animals each for norepinephrine. Data uncorrected for extraction efficiencies. Male 150 g rats were used. Animals were injected intraperitoneally (IP) with 200 mg/kg of chlordimeform (hydrochloride salt) in aqueous solution, injection volume = 0.7 ml. Control rats received 0.7 ml H_2O. Animals were sacrificed 1 hr after injection.

Biochemical Effects in Vitro: It has been already reported that chlordimeform does not inhibit cholinesterase (1). Thus, we had to look

elsewhere for the possible biochemical target system of chlordimeform.

To study the biochemical cause of chlordimeform poisoning, we have examined its effect upon the monoamine oxidase of the rat liver. Livers were homogenized in 5 volumes of cold distilled water. The homogenate was filtered through glass wool and used directly as the enzyme source. Enzyme and inhibitors were preincubated for 15 min at room temperature. Enzyme activity was defined as the amount of kynuramine metabolized in 20 min at 37°C.

The results (Table 2) indicate that chlordimeform is an inhibitor of monoamine oxidase (MAO). Also, the degrees of inhibitory potency of the chlordimeform analogs correlate roughly with those of the general in vivo toxicity of these compounds to mites.

To study the effect of chlordimeform on a cholinergic receptor, the isolated frog rectus abdominis preparation was used. Male frogs (Rana pipiens) were pithed, and the pair of rectus abdominis muscles were dissected out. The posterior tendon (origin) of the muscle was anchored to a glass rod, and the insertion (anterior) was tied to the writing arm of a smoke drum recorder. Test compounds were dissolved in eserinized frog Ringer's. The preparation was immersed in the appropriate experimental solution, and muscle contractions were recorded as changes in length by displacement of the writing arm on the rotating drum. It was found that $10^{-3}M$ chlordimeform had no effect on an eserinized muscle which was sensitive to $7 \times 10^{-7}M$ ACh (Fig. 2). Thus, chlordimeform poisoning is not mediated by cholinergic systems as far as its excitatory aspects are concerned.

In Vivo Antagonism by Reserpine: We have observed that chlordimeform acts as a reserpine antagonist in the rat, for certain symptoms. Reserpine at a dose of 10 mg/kg, IP, causes immobility, tremor, and muscle rigidity in the animal. These symptoms appear within 45 min of injection, and last at least 30 hr. Rats which were pretreated with chlordimeform (50 mg/kg, IP) 90 min prior to reserpine administration did not develop tremor, and the muscle rigidity was greatly reduced. These symptoms did not develop in the chlordimeform-pretreated rats, even after 10 hr of reserpinization. To demonstrate the antagonistic action of chlordimeform on prereserpinized rats, we injected chlordimeform (50 mg/kg) into rats 2 hr or 30 hr after reserpinization (10 mg/kg). In both cases, chlordimeform treatment was followed within 10 min by the complete disappearance of tremor and rigidity of the muscles. Such an antagonistic action of chlordimeform could be explained by a possible

Table 2. MAO inhibition by chlordimeform analogs and by known MAO inhibitors.[a]

Structure	Name	pI50[b]
benzene-CH₂-CH₂-NH-NH₂	Phenelzine	5.46 ± 0.14
pyridine-C(=O)-NH-NH-CH(CH₃)₂	Iproniazid	3.50 ± 0.08
Cl-benzene(CH₃)-N=CH-N(CH₃)₂	Chlordimeform	4.49 ± 0.05
Cl-benzene(CH₃)-N=CH-NH-CH₃	C-8520	4.60 ± 0.05
H₃-benzene(CH₃)-N=CH-N(CH₃)₂		3.85 ± 0.03
Cl-benzene-N=CH-N(CH₃)₂	C-4789	3.81 ± 0.07
benzene-N=CH-N(CH₃)₂		3.23 ± 0.01

[a]Inhibitors were added to reaction tubes in 20 μl solvent (H_2O or 95% ethanol). Control tubes received solvent alone. Preincubation was begun by addition of enzyme (0.1 ml of filtered liver homogenate). The reaction was initiated by addition of 0.3 μmole of the substrate kynuramine dihydrobromide in 20μl of H_2O.

[b]Data expressed as pI₅₀ ± standard deviation, where pI₅₀ = ± log I₅₀, I₅₀ being inhibitor concentration in mole/l. (final concentration) giving 50% inhibition. Each value is mean of three to five determinations.

central adrenomimetic activity, since tremor and muscle rigidity in reserpine treated rats has been associated with low levels of dopamine in the brain (3).

Studies in Arthropod Species

Chlordimeform has varied effects in various arthropod species, and it is not easy to come up with a typical representative study material. For instance, despite the effectiveness of chlordimeform against mite species, particularly mite eggs, little work has been done on the physiology of mites
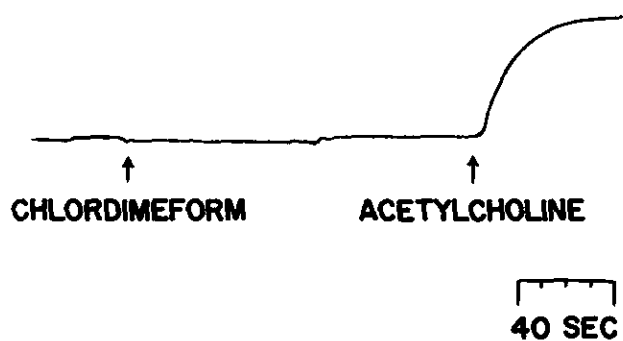
CHLORDIMEFORM   ACETYLCHOLINE

40 SEC

FIGURE 2. Muscle contractions of frog (*Rana pipiens*) rectus abdominus in response to chlordimeform ($10^{-3}M$) or AChBr ($7 \times 10^{-7}M$). The muscle origin was anchored to a glass rod, and the insertion was tied to the writing arm of a smoke drum recorder. Solutions were prepared by dissolving chlordimeform hydrochloride or acetylcholine bromide in eserinized frog Ringer's (0.002% eserine sulfate). Eserinization sensitized the muscle to ACh and prevented accommodation to repeated doses of ACh.

because of their prohibitively small size. Lepidopterous larvae are large enough, but generally unsuited for electrophysiological studies.

With respect to amine-related systems, our current knowledge in arthropod species is meager at best (4).

In the insect nervous system so far, biogenic amines, including the catecholamines norepinephrine and dopamine, and the indolamine 5-hydroxytryptamine (serotonin), have been detected, and their pharmacology has been investigated. MAO activities have been detected in cockroaches (5), grain beetles (6), European corn borers (7) and blowflies (8), but no one has yet shown the possible functional meaning of its presence.

In view of the limitations in the basic knowledge on these biochemical systems in most arthropod species, we have decided that cockroaches are the best initial study material, despite their general insensitivity to chlordimeform. Cockroaches, particularly American cockroaches, have been extensively used by physiologists and biochemists. They are most suited for electrophysiological studies. Not only that, but the American cockroach is one of two species* that has been shown to have a biogenic amine (serotonin) in the central nervous system (10–12). A basic similarity in insect and mammalian amine regulatory mechanisms can be inferred from the effects of reserpine or a MAO inhibitor on the levels of biogenic amine in the cockroach brain (13,14).

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*Catecholamines have been also detected in the brain of a locust species (9).

General Observations In the American Cockroach: Intraabdominal injection of chlordimeform hydrochloride gave an LD$_{50}$ of about 500 μg/g of body weight for American cockroaches. Injection of LD$_{50}$ is followed within 5 min by typical symptoms of intoxication. Symptoms include uncoordination, hyperactivity, arching, and wing flapping. Prostration becomes irreversible over a period of several hours, and paralysis begins in 10–20 hr. Chlordimeform did not cause tremor or twitching, and seldom induced convulsions, even at a high dose (670 μg/g). A sublethal dose of chlordimeform (420 μg/g) was followed by symptoms which lasted at least 6 hr before the insects recovered.
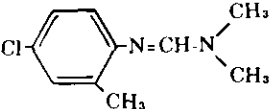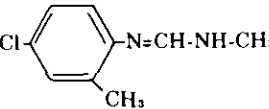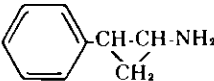
For comparative purposes, American cockroaches were poisoned with a standard MAO inhibitor, tranylcypromine hydrochloride, and the symptoms were observed. Tranylcypromine gave an LD$_{50}$ of about 700 μg/g and caused hyperactivity, uncoordination, arching, and wing flapping within 5 min of injection of the LD$_{50}$ dose. In addition to the chlordimeform like syndrome, there is another set of symptoms, including body convulsions and tremor and twitching of the appendages, which are absent in chlordimeform-poisoned insects. The usual sequence of symptoms in tranylcypromine-poisoned insects is uncoordination, hyperactivity, arching and wing flapping, soon followed by convulsions and prostration. Twitching of legs is characteristic of the prostrate insects, followed by tremor, and finally paralysis.

Preliminary experiments have established that chlordimeform does not inhibit housefly head cholinesterase even at $10^{-3}M$, nor did it affect the (Na–K) ATPase of the roach head at this concentration.

From our symptomatological observations we suspected CNS involvement in chlordimeform poisoning in American cockroaches.

Electrophysiological Studies in the American Cockroach: The effects of chlordimeform on the electrical activity of the exposed cockroach ventral nerve cord were studied. Within 10–20 min after flooding the exposed nerve with $10^{-3}M$ chlordimeform hydrochloride solution, the first electrophysiological evidence of damage to the CNS becomes apparent. This always consists of short volleys of action potentials, usually about 100 μV in amplitude. The frequency of action potentials within the volley is roughly 4/10 msec, decreasing slightly toward the end of each volley. The volleys, which usually consist of 4–7 action potentials, occur irregularly at a frequency of about 2–4/sec. The duration of each volley is on the order of 15 msec.

Table 3. Inhibition of tryptamine metabolism from combined cockroach heads and ventral nerve cords.

Compound	Structure	$I_{50} \pm SD^a$
Chlordimeform		$4.43 \times 10^{-4} \pm 0.41$
Desmethylchlordimeform		$1.08 \times 10^{-4} \pm 0.28$
Tranylcypromine		$1.41 \times 10^{-4} \pm 1.09$

[a]Data are expressed as $I_{50} \pm$ SD. Where I_{50} is the inhibitor concentration in mole/l. giving 50% inhibition of enzyme activity. Each value is the mean of three to five determinations.

Prolonged exposure (up to 2 hr) to this dose of chlordimeform results in severe hypersensitivity of the CNS, as evidenced by long trains of repetitive discharge, both spontaneous and in response to mechanical stimulation in the form of air puffing of the cerci. Synaptic blockade did not occur, even after 2 hr of exposure to 10^{-3} M chlordimeform. At $10^{-4}M$ no evidence of CNS damage could be detected.

Inhibition of Tryptamime, DOPA, and Serotonin Metabolism: Chlordimeform inhibited metabolism of ^{14}C tryptamine in the cockroach head, as shown in Table 3. Of the three compounds tested, tranylcypromine, a known inhibitor of mammalian MAO, was the most potent inhibitor. In view of the absence of detailed studies on insect MAO, an assumption was made here that tryptamine is degraded by MAO. Recent work (15; unpublished observations from this laboratory) has shown that N-acetylation rather than oxidative deamination is the predominant pathway for tryptamine in the roach brain. To shown that such an inhibition of tryptamine metabolism by chlordimeform has some physiological consequences *in vivo*, the joint actions of chlordimeform and tryptamine were investigated (Table 4). The results clearly indicate that these two chemicals act synergistically.

In addition, the effect of chlordimeform on the *in vivo* metabolism of externally applied ^{3}H-L-DOPA (a catecholamine precursor) was studied in male cockroaches. The results (Table 5) show that norepinephrine accumulates in poisoned insects to

Table 4. Potentiation of chlordimeform toxicity by tryptamine.

	Mortality, %		
	Chlordimeform + H_2O	Acetone + tryptamine	Chlordimeform + tryptamine
Trial 1	10	0	70
Trial 2	20	0	80

[a]Data are expressed as mortality 30 hr after injection of tryptamine (500 μg) or H_2O. Ten roaches were used for each combination (total, 60 roaches). Chlordimeform (100 μg/roach) was given topically with 5 μl of acetone.

a greater extent than in unpoisoned ones, in agreement with the observation of Rutschmann et al. (16) with established MAO inhibitors in the rat brain.

We have also studied the metabolic fate of ^{14}C-serotonin as it is affected by chlordimeform. The results shown in Figure 3 clearly indicate that the metabolic pattern of serotonin is altered by the chlordimeform treatment. It must be noted however, that the amount of serotonin remaining in the control is not significantly different from that in the treated animal. It was a qualitative change in the distribution of metabolites that was significant.

Histochemical Investigation in the Roach Brain: To ascertain that the basic amine regulatory mechanisms in the central nervous system of the American cockroach are similar to those found in mammalian brain, the histochemi-

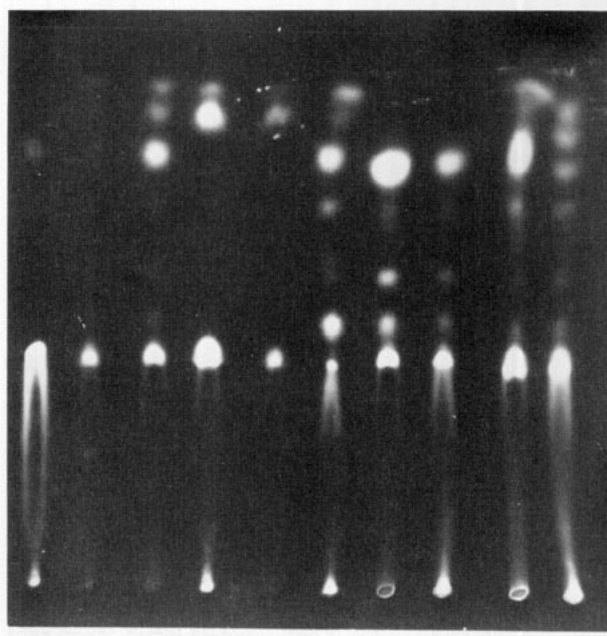|	Amounts, % of recovered radioactivity[b]	
	Control	Treated
L-DOPA	5.85	11.36
Norepinephrine	11.05	18.36
Dopamine	9.60	8.65
Other metabolites	74.17	61.75

[a]After 24 hr metabolism by male roaches. Roaches were extracted with 10 volumes of acidified n-butanol and the debris removed by brief centrifugation. The supernatant solution was extracted with 1 ml of 0.1N HCl, with 15 ml of n-hexane added to aid separation. Solvent and aqueous phases were concentrated and spotted on cellulose MN 300 TLC plates along with nonradioactive reference compounds, and the plates were developed in methanol–benzene–n-butanol–water (4:4:4:1).

[b]Results expressed in percentages of applied radioactivity (0.14 nmole of ³H-L-DOPA, specific activity 15 Ci/mmole) recovered in each fraction. Average of two determinations.

[c]Chlordimeform given topically at a dose of 100 μg/ roach 3 hr prior to the oral administration of ³H-L-DOPA. The total poisoning time for chlordimeform was 27 hr.

FIGURE 3. *In vivo* metabolism of exogenous serotonin in the adult male American cockroach. Five cockroaches were treated topically with 100 μg of chlordimeform (free base) in 5 μl acetone. Four additional roaches (controls) received acetone alone. After 2 hr, both groups were injected with 2.0 μCi of ¹⁴C-serotonin creatinine sulfate (16.2 μg) in 2 μl H₂O, and were held for an additional 24 hr. Roaches were then homogenized, each in 2.0 ml of acidified n-butanol. To account for nonmetabolic breakdown, one roach was homogenized immediately after injection of 5-HT. Homogenates were filtered through glass wool and centrifuged at 20,000 g for 10 min. A 10 μl aliquot of each supernatant fraction was spotted on silica gel HF chromatographic plates, under a constant stream of N₂. Plates were developed in darkness (solvent system used was methanol:benzene:n-butanol:H₂O, 4:4:4:1) and exposed to x-ray film for 3 weeks: (spot 1) nonmetabolizing control; (spots 2–5) untreated; (spots 6–10) chlordimeform-treated. Major spot at $R_f = 0.45$ corresponds to serotonin.

cal experiment of Frontali *(13)* was repeated. In this experiment biogenic amines were made visible by treating the freeze-dried roach brain with formaldehyde vapor. The brains were then embedded and sectioned, and the sections viewed through a fluorescence microscope. By such an approach it was possible to show that reserpine had the expected effects of depleting the amine storage in the roach brain. On the other hand, any changes in amine levels brought about by either chlordimeform or tranylcypromine (a typical MAO inhibitor) were subtle and were not detectable by such a crude, qualitative assay method (Fig. 4).

Measurment of Effects of Chlordimeform on Amine Receptors in the Cockroach CNS and Heart: To study the effects of chlordimeform on the CNS receptors for biogenic amines in the cockroach we adopted the method of Nathanson and Greengard *(17)*. This method measures stimulation of adenylate cyclase in the cockroach central nervous system as a result of the addition of exogenous biogenic amines. The sensitivity of the assay method is 1 pmole/4.5 thoracic ganglia. It immediately became apparent that it was not sensitive enough to measure the increase in the biogenic amine levels *in vivo* as a result of either MAOI or chlordimeform treatment. However, when their effects were tested *in situ* (by using isolated half ganglia) two important phenomena became known (Table 6). First chlordimeform itself does not stimulate the adenylate cyclase activity (i.e., it does

not act as a false transmitter in this preparation), and second (at 10⁻³M) it instead prevented exogenously added octopamine from achieving the maximum stimulation of adenylate cyclase activity. On the other hand, chlordimeform at 10⁻⁵M was found to increase the rate of cockroach heart beat.

Toxicities of Typical MAO Inhibitors to Cheese Mites: As mentioned before, a MAO inhibitor such as tranylcypromine has been found to be almost as toxic to the American cockroach as chlordimeform: the fact itself may be used as a supportive evidence in favor of an amine-related mode of action of chlordimeform in insects. The American cockroach is not a really susceptible
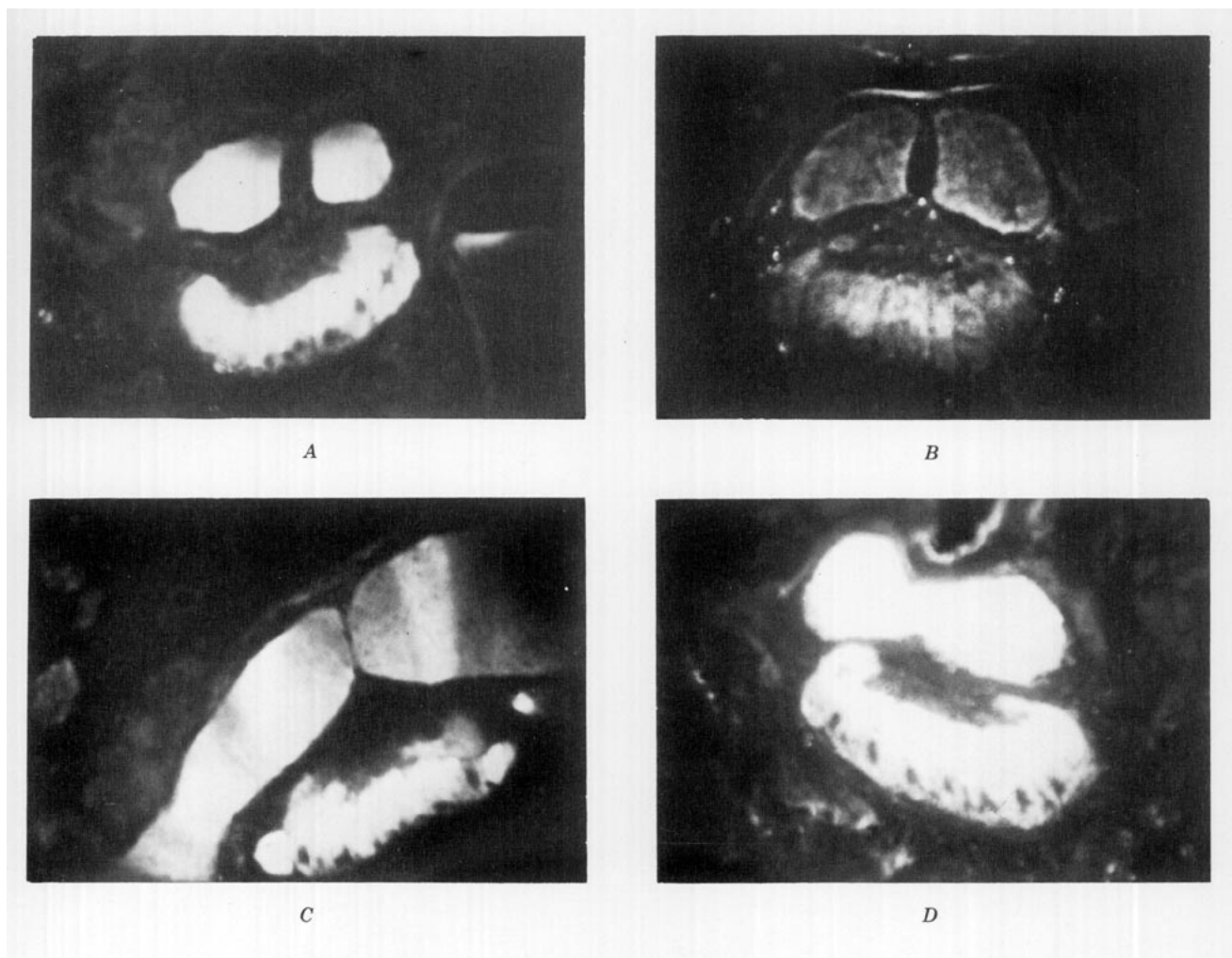
FIGURE 4. Catecholamine-induced fluorescence in brains of cockroaches (adults, male *Periplaneta americana*) receiving one of the following drug treatments: (*A*) untreated; (*B*) reserpine (50 μg); (*C*) chlordimeform (50 μg) plus DOPA (250 μg); (*D*) tranylcypromine (100 μg) plus DOPA (250 μg). Drugs were administered on each of three consecutive days. On day 4, the brains were removed, quickly frozen at Dry Ice temperature, and freeze-dried. Brains were then exposed to paraformaldehyde (equilibrated at 50% RH for 1 week) at 80°C for 3 hr, embedded in Paraplast *in vacuo*, and sectioned at 8 μm. Sections were mounted in mineral oil and viewed under fluorescence microscopy. A catecholamine-rich area of the protocerebrum (showing central body and mushroom body beta-lobes) was photographed by using Kodak high-speed Ektachrome type B film.

species for chlordimeform, however, and as such it seems appropriate to find whether MAOI are also effective against chlordimeform-susceptible species. In Table 7 we have summarized the results of our preliminary experiments on the toxicity of MAOI against the cheese mite. It can be seen that these MAOI can be good acaricides.

Tryptamine Metabolism by Homogenates of Whole Cheese Mites and Inhibition by MAO Inhibitors: To study the effect of chlordimeform on amine metabolism in a susceptible specie, we used the MAO assay technique of Wurtman and Axelrod (*18*) and applied it to whole mite homoge-

nates. Cheese mites *(Tyrophagus putrescens)* were homogenized in cold 0.25*M* sucrose at 75 mg/ml. The homogenate was centrifuged at 600*g* for 5 min, and the supernatant was used directly as the enzyme source. Assay tubes contained 0.5*M* phosphate buffer pH 7.4 (0.4 ml), distilled water (0.2 ml), and enzyme (0.1 ml). Inhibitors were added in 7 μl of distilled water, and tubes were incubated at room temperature for 15 min. The reaction was initiated by addition of ^{14}C-tryptamine (0.01 μCi, 0.21 nmole) in 10 μl of 2% EtOH. The reaction was carried out for 30 min at 37°C, and terminated by the addition of 0.7 ml of 2*N* HCl.

Table 6. Effect of chlordimeform on adenylate cyclase and octopamine—induced stimulation of adenylate cyclase in roach thoracic ganglia in situ.

Treatment	3H-c-AMP bound ± S.D., % [b]
Control	12.9 ± 0.3
Chlordimeform, $1 \times 10^{-3}M$	13.3 ±0.7
Control	15.0 ± 2.6
Octopamine, $2.5 \times 10^{-4}M$	6.4 ± 1.3
Octopamine, $2.5 \times 10^{-4}M$ +	11.6 ± 0.7
Octopamine, $2.5 \times 10^{-4}M$ + chlordimeform $1 \times 10^{-3}M$	11.6 ± 0.7

[a] Intact hemiganglia (containing adenylate cyclase) were incubated with no treatment, chlordimeform alone, octopamine alone, or octopamine and chlordimeform together, and accumulated c-AMP was measured by competitive binding to a c-AMP binding protein. Procedural details are given in Nathanson and Greengard (18).

[b] Data are expressed as % 3H-c-AMP bound ± standard deviation. All values are means of three to six determinations. Decreased binding indicates increased cyclase activity.

The toluene-extractable reaction product was measured by liquid scintillation spectrometry. The mites were found to be rich in a tryptamine-metabolizing enzyme, with product being formed at the rate of 6.7 cpm/mg whole mite/min (uncorrected for counting efficiency). The enzyme was highly sensitive to chlordimeform and to a number of MAO inhibitors (Table 8), although chromatographic analysis of the major product showed it to be different from 3-indolacetaldehyde, indole-3-acetic acid, and from N-acetyltryptamine.

Discussion

Mechanisms of Action in Mammals

There is little doubt that chlordimeform at certain concentrations (Table 2) can inhibit MAO activity. However, whether such an inhibitory property of chlordimeform by itself can account for its toxic action should be the center of discussion.

First, a consideration should be given to the fact that these so called MAOI are seldom found to be acting as pure MAO inhibitors. For instance, tranylcypromine is known to be a dangerous MAO inhibitor, since it also has some amphetamine-like action which greatly magnifies its MAO-inhibitory effects. Amphetamine inhibits re-uptake of catecholamines by specific neurons, in addition to its direct stimulating effects. Thus, often the causes of poisoning by so called MAOI are not simple.

Second in studying the action mechanisms of chlordimeform analogs, we must be also aware of

Table 7. Toxicity of MAO inhibitors to the cheese mite, Tyrophagus putrescens.[a]

Compound	Dose, μg/vial	Mortality, %
Chlordimeform	1	0
	5	100
Tranylcypromine	5	0
	20	100
SKF 9355 A	5	0
	8	50
	13	90
	20	100
SKF 9208 A	5	0
	8	10
	13	80
	20	100
Deprenyl	1	0
	3	50
	5	100

[a] Screw cap vials, 12 × 24 mm, were treated by evenly coating the inside surface with 20 μ of an acetone solution of the compound, followed by evaporation of the solvent. Ca. 50 mites (all stages) were introduced into each vial, and 12 hr mortality was estimated.

the possibility that small alterations in chemical structure may produce important changes in biological activity. For example, it has been shown that pyridyl formamidines possess adrenergic and cholinergic blocking action, whereas phenyl and naphthyl formamidines are cholinomimetics (19,20). Such consideration is important in examining the metabolic products of chlordimeform as possible active agents. With the above conditions in mind, let us proceed to weigh all the available evidence.

It is generally understood that medically used MAOI act rather slowly. MAO inhibitors also generally produce long-lasting, irreversible inhibition of MAO, so that serial doses tend to be cumulative. For example, large doses of pargyline produce a two-phase syndrome in mammals, including a fast and slow response (21). There is an initial depression of motor activity probably due to direct actions of the drug. The second phase of the syndrome is a gradual increase in activity and irritability. These slow effects develop in 4–24 hr and are probably due to catecholamine and 5-hydroxytryptamine accumulation in the central nervous system. The delayed effects of pargyline are typical of MAO inhibitors. In all species of mammal studied, most deaths from toxic intraperitoneal doses of pargyline were delayed 8–24 hr.

In contrast, the symptoms chlordimeform causes are almost reversed in time sequence. The

Table 8. Tryptamine metabolism by whole mite homogenates and inhibition by MAO inhibitors.

Compound	Structure	$I_{50} \pm$ S.D.[a]
Chlordimeform		$6.85 \times 10^{-5} \pm 0.35$
Tranylcypromine		$2.18 \times 10^{-6} \pm 0.69$
SKF 9457-A		$6.53 \times 10^{-6} \pm 0.64$
SKF 9762-A		$4.62 \times 10^{-6} \pm 0.84$
SKF 6279-A		$1.88 \times 10^{-6} \pm 0.96$
SKF 9355-A		$3.50 \times 10^{-6} \pm 1.24$
SKF 9208-A		$2.73 \times 10^{-6} \pm 2.50$
SKF 9671-A		$4.43 \times 10^{-7} \pm 0.43$
SKF 556-A		$3.00 \times 10^{-7} \pm 0.30$
SKF 10714-J		$> 10^{-4}$
SKF 9769-A		$3.32 \times 10^{-5} \pm 0.43$
Deprenyl		5.80×10^{-8b}
Lilly 51641		2.00×10^{-5b}

[a]Data are expressed as $I_{50} \pm$ S.D., where I_{50} is the inhibitor concentration in mole/l. giving 50% inhibition of enzyme activity. Most values are means of three determinations.
[b]One determination.

symptoms of hyperactivity and irritability begin immediately after injection, and depression of motor activity sets in much later stages. Death is associated with the symptoms at the early stage. Thus, it is clear that chlordimeform does not elicit a typical MAOI symptom. Rather the early violent excitation could be caused either by its direct action on amine receptors or by a yet unknown mechanism. In the former case, chlordimeform acts directly as a false transmitter (like amphetamine) and thereby stimulates the post-synaptic membrane. If such is the case, its MAO inhibitory action must play a synergistic role in the course of chlordimeform poisoning in the same way as tranylcypromine works.

The above hypothesis is consistent with our observation that poisoned rats always developed pupillary dilation within 1 hr of poisoning, indicating either direct stimulation of the receptor or stimulation of the norepinephrine (NE) releasing mechanism. Tyramine (like amphetamine) also promotes nonneural leak-out of NE, peripherally and centrally. MAO inhibitors potenitiate tyramine action by increasing extragranular NE available for leak-out (21). The possibility that direct action of chlordimeform plays a part in its poisoning processes has been suggested by us (22).

While the above symptomological observation suggests that the MAO inhibitory action of chlordimeform alone may not serve as the primary cause for the early excitation effect (and thereby its "killing action"), there are indications that such an inhibitory property of this pesticide plays a part in eliciting at least certain symptoms.

It has been noted that chlordimeform causes a hypotensive effect (i.e., sustained reduction in mean arterial pressure in the rabbit). Arterial hypotension is produced by most MAOI compounds and is generally ascribed to interference with NE liberation from peripheral sympathetic terminals, to accumulation of false transmitters such as octopamine and dopamine (both have weaker pressor action than NE), or to central effects such as the ones produced by α-methyldopa. Thus, this hypotensive effect should be regarded as indirect evidence for the MAOI-like behavior of chlordimeform.

The second evidence is that chlordimeform clearly acts antagonistically to reserpine, which is known to deplete amines from neurons by promoting leak-out from presynaptic storage sites (23). Such depletion should make a MAOI less effective because of the lack of amines to accumulate at the synapses, and because of the reserpine induced blockade of the receptor as a result of sudden flooding of synaptic areas with NE at an early stage of reserpine poisoning.

In summary, it appears that in the rat the MAO inhibitory action of chlordimeform plays a supportive part in inducing the early excitation symptoms. The primary cause of the excitation could come from its direct action. At later stages of poisoning such a MAO inhibitory action may become significant as judged by its antagonistic action to reserpine. Another effect of chlordimeform could be a blocking action on amine receptors as a result of severe chlordimeform poisoning, particularly late stages of poisoning. Such receptor blockade is expected to cause sedation. In the final analysis it is entirely possible that all these actions together constitute the unique poisoning case of chlordimeform. Thus, in studying the mode of action of chlordimeform our emphasis is the "amine-related" systems and not just MAO inhibition which likely plays only some part in chlordimeform poisonings.

Last, it is important to mention here that there is a high degree of variation among animals in the levels of amines and associated enzyme activities. For example, pargyline, when administered chronically to monkeys in large doses (100–150 mg/kg-day) produces only mild restlessness. In man, however, doses of only 3–4 mg may lead to dangerous side effects (21). Species differences could also be enormous for chlordimeform. There is danger in drawing inferences about human safety from animal data.

Mechanisms of Action in Insects and Mites

There are reasons why we separated the section on insects and mites from that on mammalian species. First, in arthropod species the functional roles of biogenic amines are not well defined, in contrast to the well studied cases in mammalian species. Second, chlordimeform acts differently in different insect, mite, and tick species. Even within one species the responses elicited by chlordimeform vary from one developmental stage to another. Third, in these arthropod species, "mode of action" is not synonymous with "mode of killing," for in many cases chlordimeform does not owe its effectiveness to direct killing action. The pests may die from exhaustion due to induced hyperexcitation (e.g., silkworm moths), drowning (e.g., rice stem borer moth), starvation (many lepidopterous larvae) due to its repellent action, loss of appetite, or behavioral changes. But these are indirect killing actions, and the true mode of action must be sought in nonlethal biochemical changes. At high doses

one can indeed induce killing, but at such concentrations many biochemical systems, other than the true target system, will be affected.

In view of such a background we must stress here that until we know much more about the underlying principles we should treat each case of poisoning separately. Even within one species, the stage of development and the tissue or systems (such as *central nervous system, malpighean tubules, respiratory system,* etc.) must be carefully specified. This is particularly important, when dealing with biogenic amine-related events, inasmuch as the physiological function of such systems might differ among the various insect and mite species or even among the various developmental stages within one species. Such evolutionary diversities are not really new. For example, glutamic acid is a CNS neurotransmitter in mammals, whereas it is a neuromuscular transmitter in insects.

In cockroaches we first of all have made efforts to study several biochemical systems that are known to be affected by conventional insecticides. A DDT or cyclodienelike mode of action for chlordimeform was ruled out because the pesticide had no effects on nerve ATPases, and because electrophysiological symptoms in poisoned nerve cords were not reminiscent of chlorinated hydrocarbonlike actions. Cholinesterase inhibition was ruled out by the difference in symptoms from organophosphate poisoning, and by the insensitivity of housefly head ChE to chlordimeform. The possibility of nicotinelike action (i.e.,direct attack on the receptor as a false transmitter) could not be adequately checked in the cockroach. However, a standard preparation of the frog rectus abdominis muscle failed to demonstrate such an action of chlordimeform. In the past, all insecticidal nicotinomimics have been tested by the same procedure (e.g., nereistoxin, Padan, etc.) and have been shown to have positive effects.

The respiratory system of the cockroach was definitely affected by chlordimeform as judged by the sharp increase in the rate of respiration (24,25) *in vivo*. A rotenone-type inhibition of coenzyme Q activity was ruled out by the lack of chlordimeform action on glutamic dehydrogenase activity in the roach (26). Its uncoupling action on oxidative phosphorylation was reported (24) and doubtlessly serves as the cause of the above respiratory changes in the roach. However, symptoms of poisoning in cockroaches are completely different from those induced by DNP, a potent uncoupler of oxidative phosphorylation. These authors themselves state that chlordimeform-induced symptoms indicate

"extensive involvement of the nervous system in poisoning" which cannot be explained by uncoupling.

As for the reports on the properties of blocking action of chlordimeform in the frog neuromuscular junction (27, 28), we believe that at least in the cockroach such an action does not play a significant role. We exposed the isolated nerve cord of the cockroach to $10^{-3}M$ chlordimeform and observed that synaptic blockade did not occur, even after 1 hr of exposure, although excitatory neurotoxic symptoms appeared after only 10 min. (26).

Thus, all of these conventional target systems being eliminated from the list, we should now carefully examine the possibility of amine-related systems being the actual target of chlordimeform. The mention of "amine-related" systems is important, since MAO inhibition may play only a part in the total picture of chlordimeform poisoning as discussed for the mammalian cases. Even well established MAO inhibitors seldom act as pure MAO inhibitors, and, furthermore, in arthropod species the role and in some cases even the presence of MAO is in doubt. For example, we could not so far demonstrate the presence of MAO in the ventral nerve cord of the American cockroach (25).

So far the direct pieces of evidence supporting the "amine-theory" in cockroaches are: (a) chlordimeform inhibits metabolism of ^{14}C-tryptamine in the roach head homogenate *in vitro* at I_{50} of 4.4 × $10^{-4}M$; (b) the killing action of chlordimeform is potentiated by tryptamine, which by itself is nontoxic to the roaches; (c) increase in indolamines levels are observed in the whole body after application of chlordimeform *in vivo*, and changes occur in metabolic patterns of serotonin *in vivo*; (d) chlordimeform at $10^{-3}M$ (we have not tested lower concentrations) blocks the stimulatory action of octopamine on adenyl cyclase in the roach thoracic ganglion; (e) chlordimeform increases the heartbeat rate in isolated roach heart preparations, the phenomenon being compatible with the report that the cockroach heart is innervated by monoamine-containing axons (29).

Indirect evidences are that tranylcypromine, a typical MAO inhibitor, produces very similar symptoms in the American cockroach. Also, several MAO inhibitors are good acaricides against the cheese mite, *Tyrophagus putrescens*.

On the other hand, there are several unanswered problems. For example; we have so far been unable to detect an increase in amine levels *in vivo* in the central nervous system of the cockroach. Also, it has recently been shown that tryptamine is not metabolized by oxidative deamination in the roach

brain, but rather by N-acetylation (15). In view of the antagonistic action of mixed-function oxidase inhibitors (such as sesamex and piperonyl butoxide), there is a possibility that one of the metabolic products, rather than chlordimeform itself, is an active agent, at least in certain species.

These questions, however, do not directly challenge the working hypothesis that the toxicity of chlordimeform in vivo is related to the changes in biogenic amine levels in quantity and/or in quality.

In conclusion, we have shown that chlordimeform can indeed affect amine regulatory mechanisms and in some instances, can react with certain amine receptors. On that basis we have proposed a working hypothesis that chlordimeform acts upon amine-related systems. Certainly much more information is needed to confirm or deny such a hypothesis. In the future, chlordimeform and its metabolic products should be tested for their effects on amine re-uptake, leak-out from the presynaptic storage, metabolism (not just MAO but many other enzymes systems) and the range of its action on amine receptors either as agonist or antagonist.

The key to the safe use of any new pesticide is to provide the basic toxicological data based upon logical explanation of its action mechanism and its side effects. Chlordimeform and its analogs and metabolites possess very peculiar and unfamiliar properties, particularly as pesticides. It appears very important to make efforts at this stage to understand the basic mechanisms of their actions. Hopefully our initial efforts are providing the means to meet the challenge.

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